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Increased oxidative stress in AOA3 cells disturbs ATM-dependent DNA damage responses

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Abstract

Ataxia Telangiectasia (AT) is caused by a mutation in the ataxia-telangiectasia-mutated (ATM) gene; the condition is associated with hyper-radiosensitivity, abnormal cell-cycle checkpoints, and genomic instability. A-T patients also show cerebellar ataxia, possibly due to reactive oxygen species (ROS) sensitivity in neural cells. The ATM protein is a key regulator of the DNA damage response. Recently, several AT-like disorders have been reported. The genes responsible for them are predicted to encode proteins that interact with ATM in the DNA-damage response. Ataxia with oculomotor apraxia types 1, 2, and 3 (AOA1, 2, and 3) result in a neurodegenerative and cellular phenotype similar to AT; however, the basis of this phenotypic similarity is unclear. Here, we show that the cells of AOA3 patients display aberrant ATM-dependent phosphorylation and apoptosis following γ-irradiation. The ATM-dependent response to H_2O_2 treatment was abrogated in AOA3 cells. Furthermore, AOA3 cells had reduced ATM activity. Our results suggest that the attenuated ATM-related response is caused by an increase in endogenous ROS in AOA3 cells. Pretreatment of cells with pyocyanin, which induces endogenous ROS production, abolished the ATM-dependent response. Moreover, AOA3 cells had decreased homologous recombination (HR) activity, and pyocyanin pretreatment reduced HR activity in HeLa cells. These results indicate that excess endogenous ROS represses the ATM-dependent cellular response and HR repair in AOA3 cells. Since the ATM-dependent cell-cycle checkpoint is an important block to carcinogenesis, such inactivation of ATM may lead to tumorigenesis as well as neurodegeneration.

1. Introduction

Double-strand breaks (DSBs) are generated in genomic DNA upon exposure to ionizing radiation (IR) or DNA-damaging agents such as bleomycin and neocarzinostatin. DSBs can also occur because of the collapse of stalled DNA replication forks. If left unrepaired, DSBs may induce genomic instability and promote apoptosis or tumorigenesis. When DSBs are recognized by cells, DNA repair factors access these sites of damage, are activated by protein kinases such as ATM, in the so-called DNA-damage response, and subsequently activate DNA repair mechanisms [1]. The study of radiation-hypersensitive genetic disorders has clarified the mechanisms of damage-induced cellular responses. One such disorder, Ataxia Telangiectasia (AT), is a rare autosomal recessive neurological condition associated with progressive cerebellar degeneration. AT is a multisystem syndrome characterized by immunodeficiency, predisposition to cancer, radiosensitivity, insulin-resistant diabetes, and premature aging [2]. In 1995, the mutated gene responsible for AT, ataxia-telangiectasia-mutated (*ATM*), was identified by positional cloning. ATM protein is activated in response to DSB damage and has a fundamental role in regulating cell-cycle checkpoints through the phosphorylation of DNA damage response (DDR) proteins including p53, checkpoint kinase 2 (Chk2), and Nijmegen breakage syndrome 1 (NBS1) [1].

Because many of the cellular and clinical features of AT and NBS overlap, the products of their causative genes are predicted to interact functionally. Bakkenist and Kastan [3] showed that radiation-induced DSBs cause intermolecular modification

within ATM dimers that leads to their activation through autophosphorylation at Ser-1981. This event triggers dimer dissociation and the free monomers subsequently phosphorylate several nuclear protein targets, which recruit DDR proteins [4]. Thus, we believe that ATM is a critical factor for the regulation of the DSB damage response.

AT is a multisystem disease that involves the nervous, immune, endocrine, and reproductive systems [5,6]. Aicardi et al. described 14 patients with slowly progressive ataxia, choreoathetosis and oculomotor apraxia [7]. The patients had an unusual form of spinocerebellar degeneration similar to AT, but they were distinguished by the later age of onset and by the sole involvement of the nervous system. This condition, now known as ataxia–oculomotor apraxia (AOA), can be subdivided into three groups, AOA types 1, 2 [8,9], and 3 (AOA1, 2, and 3) [10]. Aprataxin, the defective factor in AOA1 [11,12], is involved in DNA single-strand break repair through resolving abortive DNA ligation intermediates [13,14]. The gene product defective in AOA2 cells, Senataxin, is also implicated in the DNA damage response, particularly in response to elevated ROS [15,16]. Recently, we demonstrated that the mitochondrial cytochrome b gene is mutated in AOA3 patients. This mutation leads to mitochondrial dysfunction, resulting in an increase in endogenous ROS and aberrant ROS-induced cellular responses, including p53-dependent apoptosis [14,17]. AT patients show symptoms of neural degeneration, such as cerebellar ataxia. Thus, disruption of the ATM-dependent ROS response in AT patients may lead to neural defects, such as ataxia-oculomotor apraxia and cerebellar atrophy.

Several recent studies show that ATM plays a role in ROS-related cellular

responses. AT patients [18,19] and *Atm* knockout mice show high levels of oxidative damage [20, 21]. These data suggest that the impaired response to ROS in AT cells influences neuronal survival. Specifically, cerebellum cells exhibit low levels of NADPH [21], a major cofactor of antioxidant enzymes such as glutathione reductase and cytochrome P450 reductase. Together with superoxide dismutase and catalase, these enzymes are essential for maintenance of cellular redox balance [22]. Consistent with this finding, AT lymphoblasts reduce glutathione more slowly than do normal cells, in response to oxidative stress-induced glutathione depletion [23], possibly because of the low levels of NADPH. In fact, Cosentino et al. reported that ATM promotes p38-dependent phosphorylation of heat shock protein 27, contributing to the protection of cells against ROS accumulation through the glucose-6-phosphate dehydrogenase (G6PD)/NAPDH-dependent pathway [24]. Recently, Guo et al. showed that ROS directly induced ATM activation through the stabilization of the ATM dimers by the formation of disulfide bonds [25]. Therefore, neural degeneration in AOA3 might also be associated with defects in the ATM-related ROS response.

Here, we show that AOA3 patient cells are defective in ATM-dependent phosphorylation and subsequent apoptosis. AOA3 cells also possess reduced ATM activity and the interaction between ATM and the MRN complex is disrupted. Our data suggest that the attenuation of the ATM-related response results from increasing endogenous ROS levels. We also discuss the relationship of ATM dysfunction with ataxia-like neural degeneration and tumorigenesis.

2. Materials and Methods

2.1. Cell culture

HeLa, U2OS, hTERT-immortalized human fibroblast (48BR), hTERT-immortalized AOA3 patient-derived fibroblast (PSF-hTERT), SV40-trasformed normal fibroblast (MRC5SV), and SV40-transformed AOA3 patient-derived fibroblast (PSF-SV) [26,10] cells were cultured in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics. Normal human (GM2184, C2ABR, and C3ABR; [10, 14]), AT patient-derived (CSA [27]), and AOA3 patient lymphoblastoid cells (ATL2ABR [10]) were cultured in Roswell Park Memorial Institute medium (Sigma) supplemented with 10% FBS and antibiotics.

2.2. Irradiation

Gammacell 40 Exactor (Nordion Inc., Kanata, Canada) was used for all irradiation experiments. The radioisotope source is ^{137}Cs (132.2 TBq) and the dose rate is 0.9 Gy/min.

2.3. Antibodies

The following antibodies were used for western blot analysis or immunostaining: phospho-ATM (S1981) and γ-H2A histone family X (H2AX) mouse monoclonal antibodies (Millipore Co.); structural maintenance of chromosomes 1 (SMC1) and phospho-SMC1 (S966) rabbit polyclonal antibodies (Bethyl Laboratories Inc.); phospho-p53 (S15) mouse monoclonal, phospho-Chk2 (T68) rabbit polyclonal, phospho-H2AX rabbit polyclonal and phospho-Chk1 (S317) rabbit polyclonal antibodies (Cell Signaling Technology); human meiotic recombination 11 (hMRE11)

rabbit polyclonal, hRAD50 rabbit polyclonal; and Nbs1 rabbit polyclonal antibodies (Novus Biologicals); p53 mouse monoclonal antibody (Santa Cruz Biotechnology); Rad51 mouse monoclonal antibodies (Abnova Co.); and RPA34 (Ab-2) mouse monoclonal and H2B rabbit polyclonal antibody (Calbiochem).

2.4. Western blot analysis

Western blot analyses were carried out as described previously [26]. Target proteins were detected with the primary antibodies mentioned above and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or anti-mouse IgG antibodies (GE Healthcare). Proteins were visualized using the ECL plus chemiluminescence system (GE Healthcare).

2.5. Immunofluorescence staining

Immunofluorescence staining was carried out as described previously [26]. Alexa-488-conjugated anti-rabbit IgG or Alexa-594-conjugated anti-mouse IgG (Molecular Probes) were used to visualize the localization of the target proteins.

2.6. ATM kinase assay

The ATM kinase assay was carried out as described previously [28]. ATM was immunoprecipitated using an anti-ATM antibody (Calbiochem) from whole cell extracts. The phosphorylation of p53 (substrate) by immunoprecipitated ATM was estimated by western blot analysis using anti-phospho-p53 (S15) antibody (Cell Signaling).

2.7. HR analysis

Stable integrants for HR analysis with the DR-GFP construct in their genomic DNA were generated from normal donor (C3ABT and C12ABR) and AOA2

lymphoblastoid cell (ATL2ABR). HR analysis was performed as previously reported [29]. To measure the HR repair of I-*Sce*I-generated DSBs, I-*Sce*I expression vector (pCBASce, 50 μg) was introduced into 10^6 HeLa-DRGFP cells by electroporation (GenePulser; Bio-Rad). To determine the amount of HR repair, the percentage of GFP-positive cells was quantified 3 days post-electroporation by using the FACSCalibur™ flow cytometer (Becton Dickinson).

2.8. Propidium iodide staining to detect apoptosis

Lymphoblastoid cells from patients or normal donors were irradiated or left unirradiated. At the indicated times, cell were harvested, fixed using 70% ethanol, and then left at -20°C overnight. Fixed cells were treated with RNase (5 mg/ml) and stained with propidium iodide (PI; 50 μg/ml). The apoptotic fraction (sub G1) was quantified using a flow cytometer.

3. Results

3.1. AOA3 patient-derived cells are defective in DSB damage-induced ATM activation

Cells derived from AOA3 patients cannot increase levels of p53 protein in response to γ-ray irradiation [10], suggesting that AOA3 patient cells possess a defect in ATM activation. Previously, we found that AOA3 lymphoblastoid cells (ATL2) and A-T cells (CSA) failed to upregulate p53 protein following irradiation, while normal cells (C3ABR) elevated p53 at 4 h post-irradiation (Fig. 1A). ATM-dependent phosphorylation of p53 at S15 and SMC1 at S966 was also defective in patient lymphoblastoid cells (Fig. 1C and Fig. S1A). As expected, autophosphorylation of ATM,

a hallmark of ATM activation, occurred in normal cells but not in AOA3 cells (Fig. 1A and Fig. S1A). NBS1, MRE11, and RAD50 are essential for DSB damage-induced ATM activation; expression of these factors in AOA3 cells was normal (Fig. 1A). We also examined irradiated AOA3 patient-derived fibroblasts (Fig. 1B). These cells (PSF-SV) exhibited neither ATM autophosphorylation nor ATM-dependent phosphorylation of SMC1 and histone H2AX (Fig. 1B). Although ATM/p53-dependent apoptosis can be induced in Epstein-Barr (EB) virus-transformed lymphoblastoid cells [30], the number of apoptotic cells was less than that observed in normal cells (Fig. 1D). Caspase 3 is normally activated as part of the apoptosis pathway; however, caspase 3-dependent cleavage of PARP was not detected in AOA3 patient cells (ATL2ABR; Fig. 1C). In the case of SV40-transformed fibroblasts, AOA3 (PSFSV) increased apoptosis after irradiation more than normal cells, similarly to AT fibroblasts (Fig. S2B). These data suggest that AOA3 cells cannot activate ATM and its associated pathways in response to DSB damage.

3.2. AOA 3 patient-derived cells lack oxidative stress-dependent ATM activation

Recently, Guo et al. reported that ATM is activated in vitro and in vivo following H_2O_2 treatment [25]. Other reports also suggest that oxidative stress induces the activation of ATM [31,32]. Figure 2A shows that H_2O_2 treatment at concentrations above 50 μM triggered autophosphorylation of ATM and ATM-dependent phosphorylation of SMC1 and Chk2 in normal lymphoblastoid cells (C2ABR), whereas AOA3 patient cells (ATL2ABR) showed decreases in SMC1 and Chk2 phosphorylation,

particularly at lower H_2O_2 concentrations (Fig. S1BC). In western blots, the delayed mobility of the NBS1 band indicated that it was phosphorylated. A delayed NBS1 band was not present in lysates of AOA3 cells treated with H_2O_2 (Fig. 2A). AOA3 patient cells showed basal autophosphorylation of ATM, but this did not increase with H_2O_2 treatment. Phosphorylation of ATM was observed following H_2O_2 treatment in normal cells (MRC5SV), but the phosphorylation was remarkably less in patient cells (PSF-SV). EB virus transformation of lymphoblastoid cells induced ATM/p53-dependent apoptosis. However, ATM/p53-dependent apoptosis was not induced in transformed AOA lymphoblastoid cells. We also confirmed the apoptotic defect by examining PARP degradation. H_2O_2 treatment did not induce the cleavage of PARP in AOA3 cells (Fig. 2C). Using PI staining, we confirmed repression of apoptosis in AOA3 cells (Fig. S2A). On the other hand, AOA3 fibroblasts as well as AT fibroblasts increased apoptosis following H_2O_2 treatment, compared with normal cells (Fig. S2C). These results suggest that AOA3 cells are also defective in oxidative stress-induced ATM activation. Oxidative stress-activation by the oxidizing agent diamide reportedly induces the homodimerization of ATM through the formation of disulfide bonds that subsequently activate ATM [25]. Diamide treatment induced autophosphorylation of ATM and subsequent phosphorylation of Chk2, p53, and SMC1 in normal cells (MRC5SV), but only slightly increased the phosphorylation of these factors in patient cells (Fig. 2D). Thus, ATM might form abnormal structures in AOA3 cells, leading to its inactivation.

3.3. Endogenous ROS disrupts the interaction between ATM and the NBS1 complex

As shown in Fig. 1 and 2, ATM activity was abolished in AOA3 patient cells. Next, we verified the activity of ATM by using an in vitro kinase assay (Fig. 3A). Anti-ATM immunoprecipitates from irradiated cell extracts phosphorylated the p53 substrate. H_2O_2 treatment also induced ATM kinase activity in vitro. However, the immunoprecipitates from AOA3 cells did not show ATM activity under any of the treatment conditions, consistent with the results shown in Fig. 1 and 2. Because the interaction of ATM and NBS1, and the subsequent recruitment of this complex to DSB damage sites are indispensable for sufficient activation of ATM [33], we examined this interaction in AOA3 patient-derived cells (Fig. 3B). In normal cells, the anti-ATM antibody coprecipitated NBS1 after irradiation. However, NBS1 could not be immunoprecipitated from irradiated AOA3 cells (ATL2ABR). As shown in Fig. S3A, phosphorylated ATM showed broad band in AOA3 cells (PSF), suggesting abnormal structure formation of ATM. This abnormal structure may disturb interaction between ATM and NBS1. Notably, oxidative stress did not stimulate the interaction between ATM and NBS1 in either normal or AOA3 cells (ATL2ABR; Fig. 3B). The absence of this interaction is in agreement with a previous report demonstrating that the MRE11/NBS1/RAD50 complex is dispensable for ROS-induced activation of ATM [25]. We also verified the interaction between ATM and NBS1 by immunofluorescence (Fig. 3C and Fig. S3B). In more than 80% of hTERT-immortalized normal fibroblasts (48BR), there was a clear colocalization of phospho-ATM and NBS1 following irradiation (5 Gy). Colocalization was not observed in more than 50% of patient fibroblasts (PSF-SV) treated under the same conditions. We speculate that abolishing the interaction between

ATM and NBS1 leads to a deficiency in ATM activation in AOA3 cells.

We recently reported that AOA3 cells show increased endogenous ROS levels, attributed to mitochondrial defects (Fig. S4) [17]. The increase in ROS levels may disrupt ATM activation and its associated responses. Pyocyanin, an electron receptor, disrupts the electron transport chain in mitochondria, leading to an increase in endogenous ROS. Pyocyanin treatment increased superoxide levels in normal cells (Fig. S6 and S7). γ-Ray irradiation (5 Gy) induced the phosphorylation of ATM and Chk2 in U2OS cells, but pyocyanin pretreatment suppressed the phosphorylation of these proteins (Fig. 3D). Therefore, we predict that the increase of endogenous ROS in AOA3 cells prevents ATM activation in response to DNA damage.

3.4. Endogenous ROS disrupts homologous recombination repair

Kinases of the ATM family participate in DSB repairs via the non-homologous end-joining (NHEJ) and homologous recombination (HR) pathways. Among these kinases, DNA-dependent protein kinase catalytic subunit is an important regulator of NHEJ repair, and ataxia-telangiectasia and Rad3-related protein is reported to contribute to HR through the phosphorylation of Chk1 or other substrates [34]. ATM also participates in HR repair through the phosphorylation of H2AX protein and mediator of DNA damage checkpoint protein 1 (MDC1) [35,36]. Therefore, the increase in endogenous ROS may disrupt HR repair in AOA3 cells. We investigated whether AOA3 cells formed Rad51 foci, a marker for the initial step in the HR pathway, following irradiation (Fig. 4A and Fig S5). More than 20% of the normal cells (48BR)

possessed Rad51 foci 4 h after irradiation (5 Gy). However, Rad51 foci did not form in AOA3 cells (PSF) 4 h post-irradiation, suggesting a defect in HR in patient cells. To estimate HR activity, we used a DR-GFP reporter system [29]. HR repair of DSBs generated by I-*Sce*I induces the cells to express GFP. HR activity can then be estimated by calculating the percentage of GFP-positive cells, quantified using a flow cytometer. To estimate HR activity in AOA3 cells, we generated normal donor and patient cell lines that possess the DR-GFP construct and then counted the number of GFP-positive cells, after introducing the I-*Sce*I expression vector (Fig. 4B). Cultures of normal donor-derived cells (C3DR#1 and C12DR#2) contained approximately 5% GFP-positive cells, but this number decreased by more than half in patient cells (ATL2DR#10, ATL2DR#12). These results indicate that patient cells have reduced HR activity.

As reported previously, AOA3 cells have increased endogenous ROS levels [17]. Figure 3D shows the increase in endogenous ROS by pyocyanin-repressed ATM-dependent phosphorylation. Thus, we investigated whether endogenous ROS also reduced HR activity (Fig. 4C). Approximately 10% of HeLa-DRGFP cells were GFP-positive after the introduction of I-*Sce*I, but pyocyanin pretreatment decreased this to approximately 5%. However, NHEJ activity was not reduced by pyocyanin pretreatment (Fig. S5B). We speculate that the excess of endogenous ROS represses the HR repair pathway in AOA3 patient cells.

4. Discussion

AOA3 and AT patients show similar neural degeneration phenotypes. AOA3-derived cells also have impaired induction of p53 in response to DSB damage [10]. Because the mutation associated with AOA is in cytochrome c, and the *ATM* gene in AOA patients is functional [17], it is unclear why AOA3 patients display p53-associated defects and an AT-like neural phenotype. Here, we demonstrate that AOA3 patient-derived cells are defective in ATM autophosphorylation and phosphorylation of ATM substrates following irradiation or H_2O_2 treatment (Fig. 1A, B and Fig. 2 A, B). ATM/p53-dependent apoptosis was also abolished in AOA3 cells (Fig. 1C, D and Fig. 2C). ATM kinase activity is altered in AOA3 cells (Fig. 3A). Furthermore, our results suggest that ATM kinase is inactivated in AOA3 cells; that ATM cannot interact with NBS1 in irradiated AOA3 cells (Fig. 3BC); and that ATM is unable to dimerize and become active in response to diamide treatment (Fig. 3D). Moreover, pyocyanin-induced increases in endogenous ROS abrogated the phosphorylation of ATM and its substrates (Fig. 3D). These results suggest the following mechanism of ATM inactivation in AOA3 cells (Fig. 5). AOA3 cells increase endogenous ROS levels resulting from mitochondrial defects caused by the cytochrome c mutation, which could lead to abnormal homodimer formation of ATM through disulfide bonds. As a result, ATM cannot be activated by increased ROS and generation of DNA double-strand breaks (Fig. 5).

We also showed that excess levels of endogenous ROS reduced DSB-induced activation of ATM in vivo. Specifically, an increase in endogenous ROS by pyocyanin treatment reduced DSB damage-induced activation of ATM in vivo (Fig. 3D). Few studies have

directly addressed whether excess ROS influences ATM activation; however, the effect of H_2O_2 treatment on in vitro ATM activity has been reported [25]. In that study, the excess of H_2O_2 did not disrupt the interaction between ATM and the MRN complex, but it did appear to reduce MRN-dependent ATM activity in vitro. Excess H_2O_2 also reduced the binding of MRN with DNA. Therefore, excess ROS in AOA3 cells might also reduce ATM activation by inhibiting the MRN complex from binding to DSBs following irradiation. Although the report by Guo et al. showed that H_2O_2 treatment did not inhibit the interaction between ATM and the MRN complex [37], DSB damage-induced interaction was diminished in AOA3. Our previous work indicated that superoxide rather than H_2O_2 was elevated in AOA3 cells [17]. In this study, pyocyanin was found to increase endogenous superoxide, but H_2O_2 treatment did not (Fig. S6 and S7). Furthermore, pyocyanin treatment attenuated ATM activation in U2OS cells (Fig. 3D), but H_2O_2 treatment did not. Therefore, we predict that the increase in endogenous superoxide interferes with ATM activation through disrupting the interaction between ATM and the MRN complex.

We also speculate that the excess of endogenous ROS impairs HR repair. AOA3 cells reduced DSB damage-induced Rad51 foci formation and the DR-GFP assay showed that HR activity was reduced in AOA3 cells (Fig. 4A, B). In fact, the pyocyanin-induced increase in ROS also reduced HR activity according to the DR-GFP assay (Fig. 4C). The results of several studies suggest that ATM plays a role in the HR pathway [38-40]. The ATM-dependent phosphorylation of factors such as MDC1 and H2AX is important for the H2A/H2AX ubiquitination pathway and contributes to

recruitment of several HR factors [35,36]. The reduction of HR in AOA3 cells was attributed to inhibition of ATM activity by endogenous ROS in those studies. However, recent work shows that ATM is dispensable for HR repair in cells of various murine tissues [41], suggesting that inactivation of ATM by endogenous ROS is not critical for repression of HR repair. Guo et al. reported that excess ROS abolished the interaction between the MRN complex and double-strand DNA [37]. Because the MRN complex is critical for the initial step of HR repair, disrupting this interaction may inhibit the HR repair pathway in AOA3 cells. Alternatively, endogenous ROS may activate the stress-activated protein kinase (SAPK) pathway. Recently, the SAPK-dependent phosphorylation of Rad52 was shown to be indispensable for the regulation of HR in fission yeast [42]. Therefore, modulation of SAPK or other ROS-responsive enzymes is likely to affect the mammalian HR pathway.

ATM plays a critical role in the cellular response to oxidative stress [24,25,43]. ATM-deficient human or mouse cells have increased levels of endogenous ROS and accumulate oxidative damage [18-21]. H_2O_2 treatment activates ATM both in vitro and in vivo [25], and ATM is indispensable for protection from ROS accumulation through the G6PD/NAPDH-dependent pathway [24]. ATM-deficient neural stem cells have reduced p38 mitogen-activated protein kinase (MAPK)-regulated proliferation. However, the antioxidant N-acetyl-L-cysteine or p38MAPK inhibitor can restore normal proliferation in these cells [44-46]. These findings indicate that the antioxidant function of ATM is essential for the proliferation of neural stem cells, suggesting that neural degeneration in AT patient results from a deficiency in the ATM anti-oxidative

response. As we showed here, ATM-related responses in AOA3 patient cells could be disturbed. This disturbance may influence ATM-related responses with oxidative stress in neural cells and lead to neurodegeneration phenotype in AOA3 cell. In order to clarify the detailed mechanisms of neurodegeneration and develop effective therapies for AOA3 or similar disorders, we must further evaluate the functional integrity of ATM in mitochondrial disorders such as AOA3 and other ataxia-associated diseases.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1. Oculomotor apraxia Type 3 (AOA3) cells have aberrant ataxia-telangiectasia-mutated (ATM)-dependent responses following irradiation. A–C: Normal (C3ABR), Ataxia Telangiectasia (AT; CSA), and AOA3 (ATL2ABR) lymphoblastoid cells, or normal (MRC5SV) and AOA3 (PSF-SV) fibroblasts were γ-ray-irradiated (5 Gy), harvested at the indicated times post-irradiation, and analyzed by western blot using the indicated antibodies. D: Normal (C3ABR), AT (CSA), and AOA3 (ATL2ABR) lymphoblastoid cells were γ-irradiated (5 Gy) and harvested at the indicated times post-irradiation. The cells were fixed in ethanol and stained with propidium iodide (PI). The percentage of apoptotic cells (sub G1) was quantified using a flow cytometer.

Fig. 2. AOA3 cells are defective in the ATM-dependent response following H_2O_2 treatment. A: Normal (C2ABR) and AOA3 (ATL2ABR) lymphoblastoid cells were treated with the indicated concentrations of H_2O_2 for 1 h. Harvested cells were analyzed by western blot using the indicated antibodies. B and C: Normal (C2ABR) and AOA3 (ATL2ABR) lymphoblastoid cells, or normal (MRC5SV) and AOA3 (PSF-SV) fibroblasts were treated with 100 μM of H_2O_2 for 1 h. After washing out the H_2O_2, the cells were incubated for the indicated times. Harvested cells were then analyzed by western blot using the indicated antibodies. D: Normal (MRC5SV) and AOA3 (PSF-SV) fibroblasts were treated with diamide (100 μM) for the indicated times. Harvested cells were analyzed by western blot using the indicated antibodies.

Fig. 3. AOA3 cells reduce ATM activity by disrupting the interaction between ATM and Nijmegen breakage syndrome (NBS1). A: Normal (C2ABR) or AOA3 (ATL2ABR) lymphoblastoid cells were γ-irradiated (10 Gy) or treated with H_2O_2, 100 μM. After 1 h, cells were harvested and a kinase activity assay was performed by generating immunocomplexes using an anti-ATM antibody. Phosphorylation was detected by western blot analysis using an anti-phospho-p53 antibody. The amount of substrate was confirmed using an anti-GST antibody. B: Extracts from normal (C2ABR) or AOA3 (ATL2ABR) lymphoblastoid cells were immunoprecipitated with anti-ATM antibody, and the immunocomplexes were detected by western blot analysis using an anti-NBS1 antibody. C: Normal (48BR) and AOA3 (PSF-SV) fibroblasts were γ-irradiated (5 Gy). After 30 min., the cells were fixed and immunostaining was performed using anti-phospho-ATM and anti-NBS1 antibodies. DAPI staining (blue) was used to counterstain the nuclei. (D) U2OS cells were pretreated with pyocyanin (50 μM) for 24 h. The cells were γ-irradiated (5 Gy) and harvested at the indicated times post-irradiation. Cell lysates were then analyzed by western blot using indicated antibodies.

Fig. 4. AOA3 cells reduce homologous recombination repair activity.

A: Normal (48BR) and AOA3 (PSF-hTERT) fibroblasts were γ-irradiated (5 Gy). After 4 h, the cells were fixed and immunostaining was performed using an anti-Rad51 antibody. Rad51 foci-positive cells were counted under a fluorescence microscope. B:

AOA3 lymphoblastoid cells with reduced HR activity. I-*Sce*I expression plasmids were electroporated into normal or AOA3 lymphoblastoid cells, carrying DRGFP construct in their genome. After 3 d, GFP-positive cells, which have an activated HR pathway, were analyzed using a flow cytometer. C: HeLa-DRGFP cells were pretreated with pyocyanin (50 μM) for 24 h. I-*Sce*I expression plasmids were introduced into cells carrying the DR-GFP construct in their genome. After 3 d, GFP-positive cells were analyzed using a flow cytometer.

Fig. 5. Excess endogenous ROS inactivate ATM in AOA3 cells. In normal cells, increases in exogenous or endogenous ROS trigger dimerization of ATM as active form through a disulfide bond. However, in the case of AOA3 cells, excess endogenous ROS make ATM form abnormal dimers through many disulfide bonds. As a result, ATM cannot be activated by ROS and DNA damage. Such inactivation of ATM could lead to neurodegeneration, gene mutation and genomic instability.

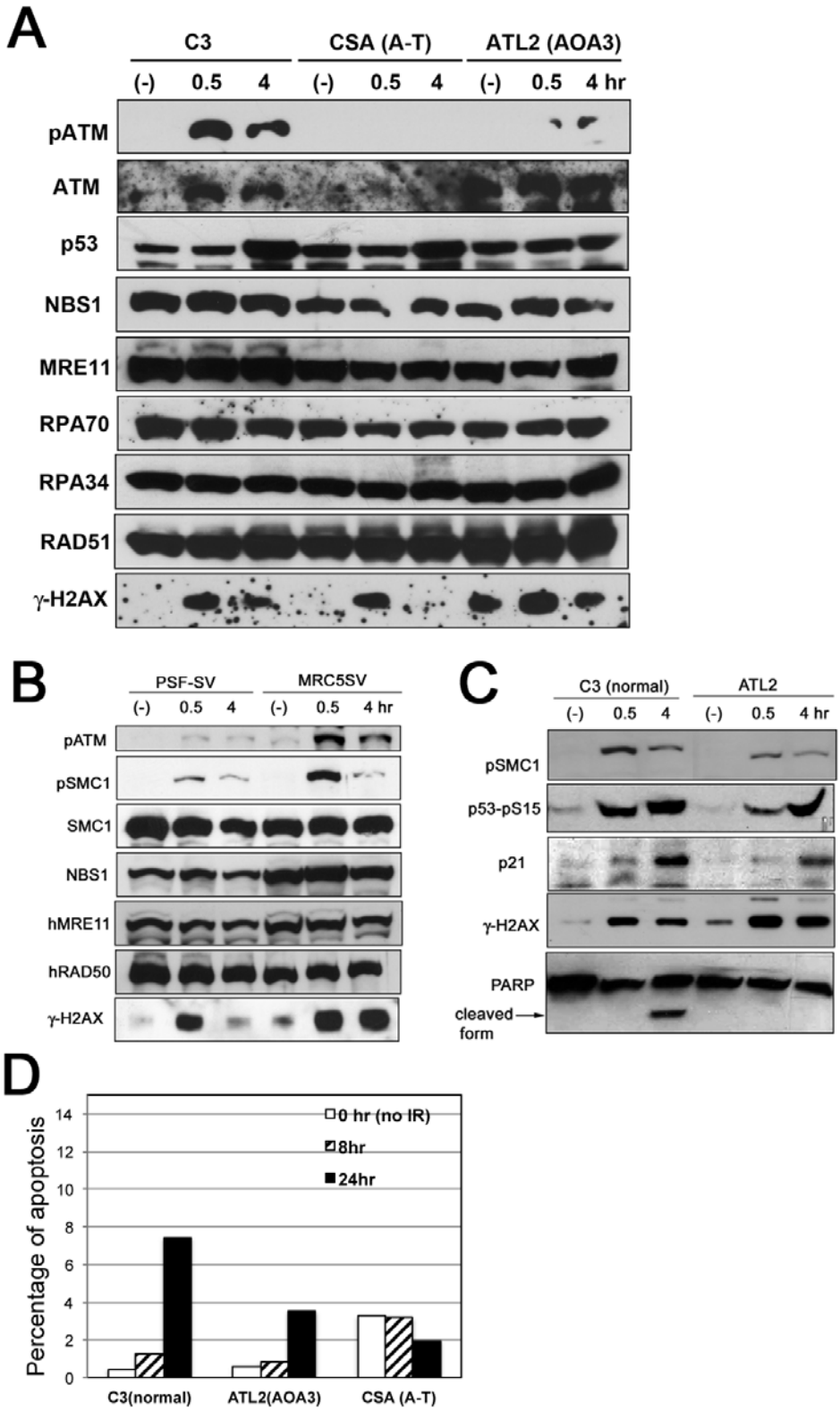
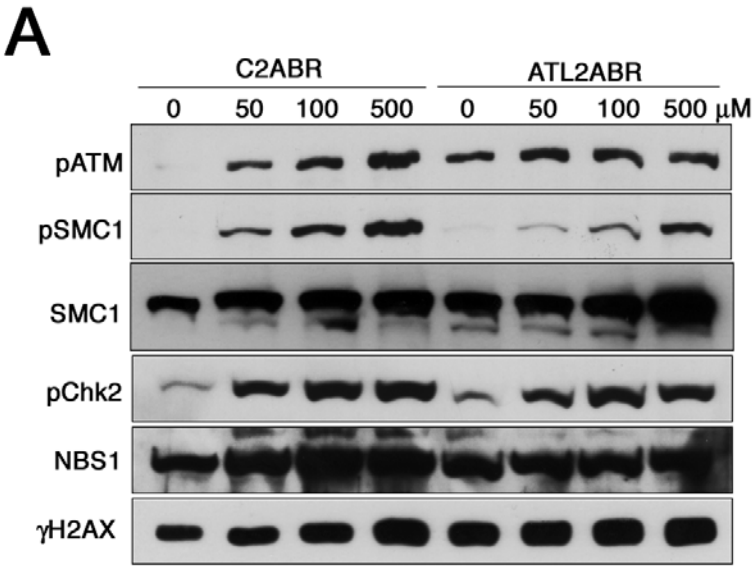
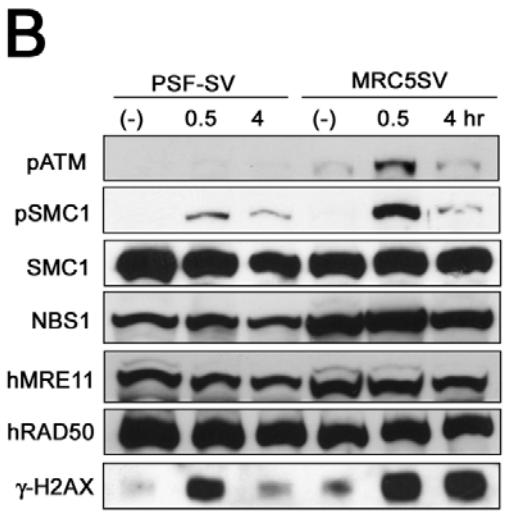
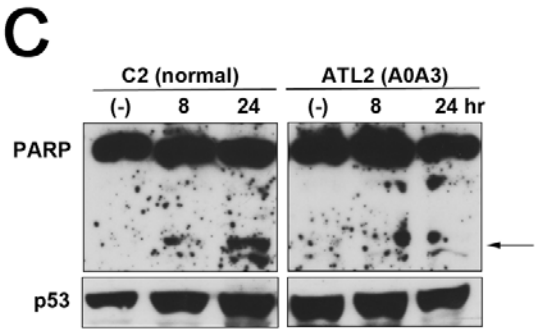


Figure 1







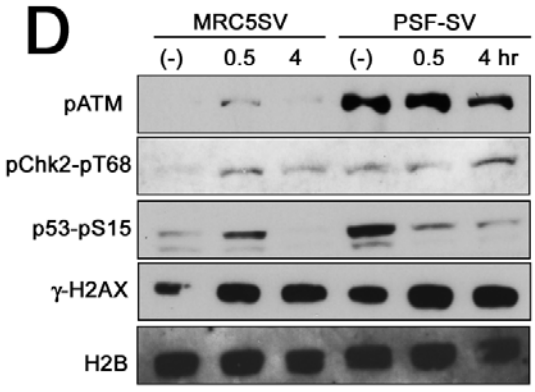


Figure 2

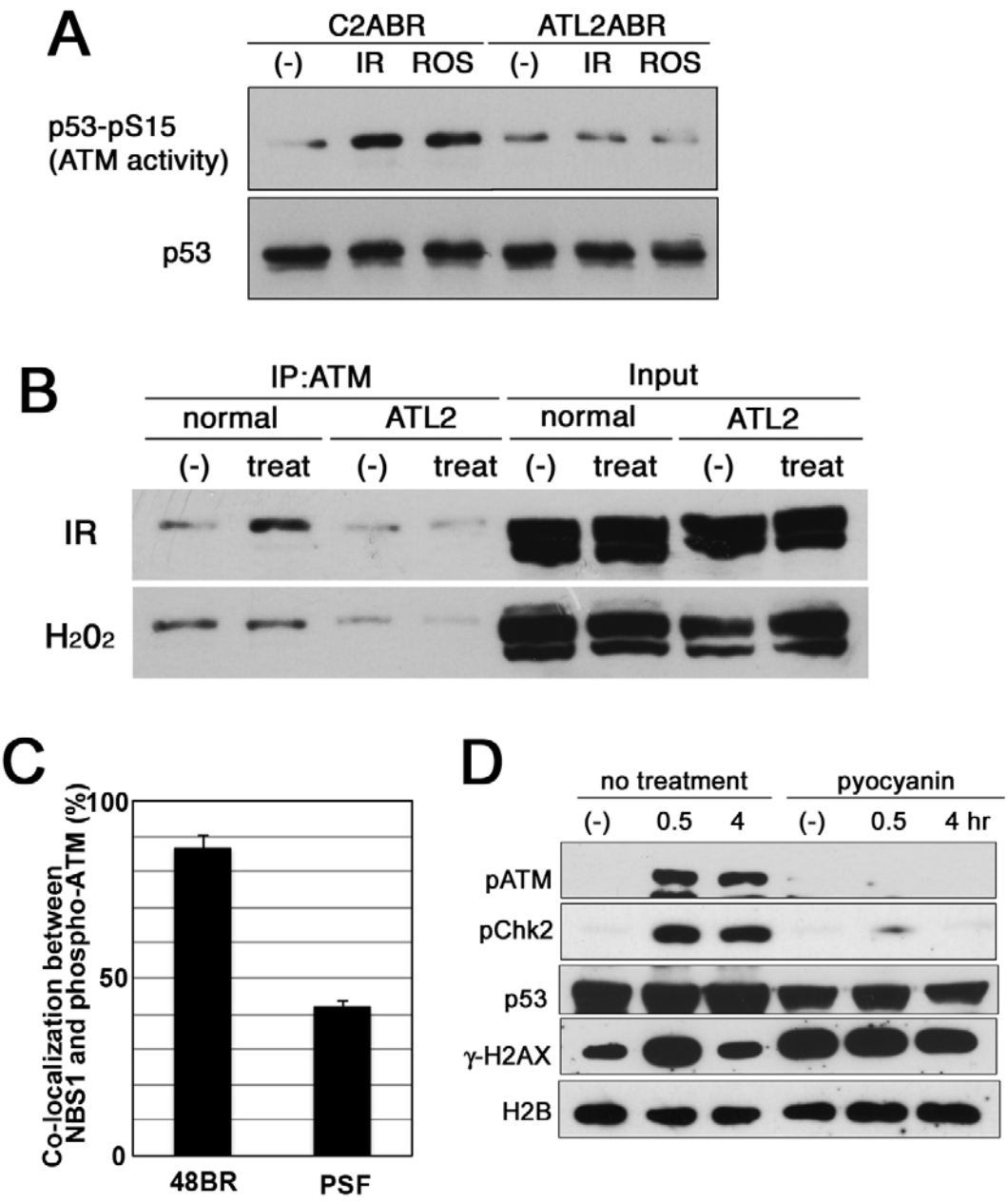


Figure 3

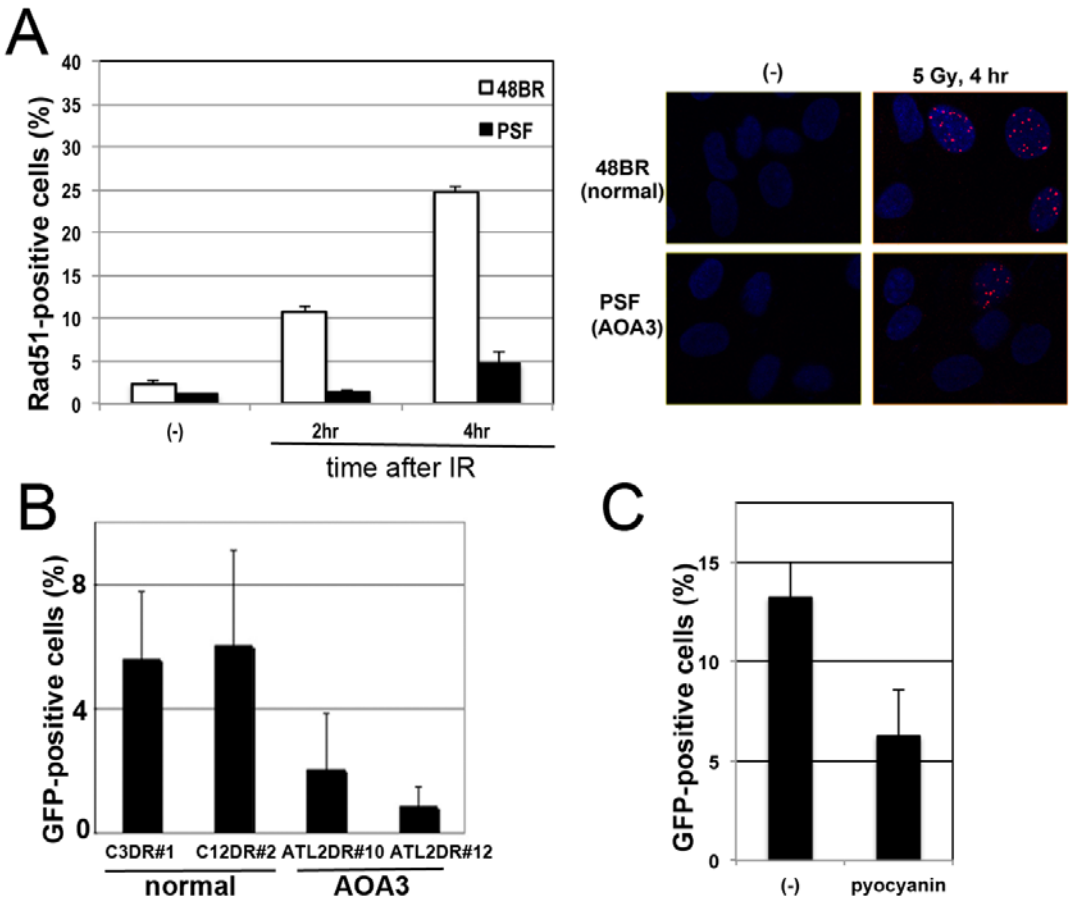


Figure 4

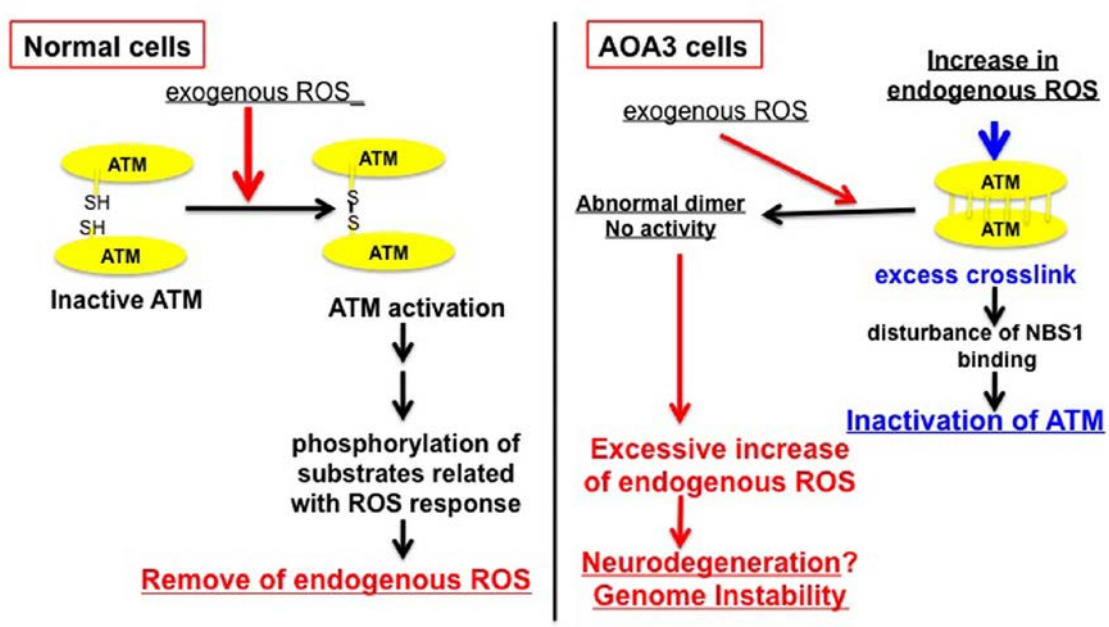


Figure 5